***Selenastrum capricornutum* 96-Hour Toxicity Test**

1.0 OBJECTIVE

In laboratory tests designed to determine the toxicity of low-salinity water samples, the unicellular alga *Selenastrum capricornutum* is exposed to ambient samples for 96 hours, after which the cell growth is determined in each toxicant concentration. Observed effects may be related to the presence of contaminants or to naturally occurring factors. In order to correctly interpret toxicity results, concentrations of chemical contaminants should be analyzed, as well as other water quality parameters, such as pH, dissolved oxygen, hardness, alkalinity, temperature, ammonia and conductivity.

In this procedure, water samples collected from field stations are divided into randomly numbered replicate test containers in the laboratory. A known cell density of *Selenastrum* is placed into each replicate container and monitored for growth. Because the test measures effects on an early life-stage of an ecologically important species possessing relatively stringent water quality requirements, the results constitute a good basis for decisions concerning either hazard evaluation or the suitability of ambient waters for aquatic life (US EPA, 2002).

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate standard operating procedure (MPSL SOP 1.3).

2.1 Culture

- Pipettes, tubing, and clean air system
- 2-liter Erlenmeyer flasks
- 4:1 water prepared from Nanopure® and Evian® (25 ± 1°C)

2.2 Test Initiation/Termination

- Environmental chamber (25 ± 1°C, continuous illumination at 86 ± 8.6 $\mu E/m^2/s$)
- 125-mL clean Erlenmeyer flasks (5 per sample, 3 per reference toxicant dilution), with covers
- 1000-mL volumetric flask for reference toxicant dilutions
- 10-mL and micropipettors and pipettes for reference toxicant dilutions
- Cupric chloride stock solution (10,000 µg/L Cu)
- Randomization sheet to arrange and identify test containers
- Data sheets
- Gloves and appropriate safety gear (see MPSL lab safety manual)

• Sample vials for reference toxicant analysis (new polyethylene 60 mL)

• Spectrophotometer with disposable cuvettes

2.3 Water Quality

• Meters and probes for measuring pH, dissolved oxygen, hardness, alkalinity, ammonia, and conductivity

• Thermometers (glass spirit thermometer and continuously recording thermometer)

• Graduated pipettes and hand pipette pump for water quality sampling

• Gloves and appropriate safety gear (see MPSL lab safety manual)

2.4 Dilution Water

In every step of this procedure, use Granite Canyon Nanopure® water mixed with Evian® in a 4:1 ratio. This water is then nutrient enriched as described below. Conductivity should not exceed 3000 μS/cm at any time. Hardness (as $CaCO_3$) should not exceed 700 mg/L.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate test flasks for each sample concentration (four to be analyzed for growth and one for daily water quality). Flasks are arranged randomly, and each receives a known cell concentration. The quality of *Selenastrum* and testing conditions is determined through concurrent testing of reference toxicants (positive controls) and control water (negative controls). Testing of reference sites or receiving water is recommended to demonstrate the suitability of test sites in the absence of toxic contaminant concentrations. Test conditions of temperature and photoperiod are controlled as indicated below, and dissolved oxygen, pH, conductivity, and NH_3 are measured at the beginning of the exposure. Temperature and pH are measured daily. Temperature is measured continuously, and hardness and alkalinity are measured at the beginning of the test.

4.0 PREPARATION OF SAMPLES FOR TESTING

Because of the 48-hour holding time, tests will generally be initiated on the same day as sample receipt. Filter sample through a 25-μm screen and place appropriate sample volume in the constant temperature room. Allow oxygen concentrations to equilibrate below super-saturated levels. Aerate if super saturated. There are five nutrients that get added to each sample at a concentration of 1mL nutrient/L sample. Nutrient recipes are contained

in the laboratory recipe notebook. Prepare five replicate flasks for each sample to be tested. Consult the random number sheet to ensure proper randomization. Each container receives 25 mL of test solution.

5.0 CONTROLS

5.1 Dilution Control

The dilution control consists of 4:1 culture water that has received a nutrient boost.

5.2 Reference Toxicant Test

For cultured organisms, conduct a concurrent reference toxicant at least monthly. The reference toxicant test indicates the sensitivity of the organisms and the suitability of the test methodology.

Reagent grade cupric chloride ($CuCl_2$) should be used as the reference toxicant for *Selenastrum* tests, unless the Regional Water Quality Control Board or other appropriate regulatory agency specifies another toxicant. Prepare a 10,000 µg/L Cu stock solution by adding 0.0268g of reagent grade $CuCl_2$ to a final volume of one liter of distilled water in a plastic volumetric flask. Cap tightly and mix thoroughly. Sample and log the reference toxicant stock solution at the beginning of the test for chemical verification of the copper concentration. Acidify samples for analysis in clean sample vials with 1% by volume 14N-reagent grade nitric acid.

Reference toxicant solutions should be three to five replicates of 0 (control), 10, 18, 32, 56, and 100 µg Cu/L. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare each concentration according to the dilution schedule using nutrient enriched 4:1 water. Aliquot each concentration to randomly numbered test containers as indicated on the random number sheet, and into water quality vials. Start with the control solutions and progress to the highest concentration to minimize contamination. Allow reference toxicant test containers to equilibrate in the constant temperature room.

6.0 TEST INITIATION

Prepare cell inoculum by determining the cell density of the stock culture (4 – 7 days old; purchased from Aquatic Bio Systems). Using the test initiation data sheet, follow the equations for determining the volume of inoculum. The final concentration of cells in the flasks should be 10,000 cells/mL. Measure temperature, dissolved oxygen, pH, conductivity, ammonia, alkalinity , and hardness in each sample at the beginning of the test. Sample the initial test solutions at the time of sample preparation.

7.0 MONITORING THE TOXICITY TEST

Flasks are shaken twice daily in the morning and afternoon. During the shaking process, flasks should be re-randomized to ensure equal exposure to the light regime. If a test s to be conducted over a weekend, flasks should be shaken at the start and end of the shift. Temperature and pH are measured and recorded daily from the water quality flask for each site.

8.0 TERMINATING THE TOXICITY TEST

After 96 hours of exposure cell density in each flask is determined using a hemacytometer or UV spectrophotometer set to 750 nm. If using the spectrophotometric method, the instrument should be blanked with each sample. Final water quality must be sampled at the termination of the test.

Take the completed data sheet to the office for data entry and analysis. Notify the data analyst that the data has arrived. Make sure the data sheets are placed in the proper location and that the person keeping track of the data knows where it is.

9.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, check the data sheet to determine whether dilution water and conductivity controls have acceptable survival (cell growth >1,000,000 cells/mL). Tests with temperature, conductivity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are 25 ± 1°C; acceptable dissolved oxygen concentration is 60-100% saturation.

10.0 REFERENCES

U.S. Environmental Protection Agency. 2002. Short-term methods for estimating the chromic toxicity of effluents and receiving water to freshwater organisms. EPA-821-R-02-013. Office of Research and Development. Washington, DC.

11.0 TEST SUMMARY

Species:	*Selenastrum capricornutum*
Test Duration:	96 hours
Endpoint:	cell growth
Organism Source	Aquatic Bio Systems (Fort Collins, CO)
Age of Test Organisms:	4 to 7 days
Temperature:	25 ± 1°C recommended (range not to exceed 3°C required)

Selenastrum capricornutum 96-Hour Toxicity Test

Dissolved Oxygen	4 mg/L recommended
Dilution Water:	Evian®:Nanopure® 4:1 with nutrients
Light intensity:	Soft fluorescent illumination (86 ± 8.6 µE/m^2/s)
Photoperiod:	constant light
Replication:	4 (samples), 3 (reference toxicant)
Test Containers:	125-mL erlenmeyer flasks
Test Solution Volume:	25 mL
Loading:	10,000 cells/mL
Reference Toxicant:	copper chloride ($CuCl_2$)
Daily Monitoring:	shake twice, measure pH and temperature
Acceptability Criteria:	cell growth > 200,000 cells/mL